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MICE CAN RECOVER FROM PULMONARY INFLUENZA VIRUS INFECTION IN THE ABSENCE OF CLASS I-RESTRICTED CYTOTOXIC T CELLS[1]

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Intranasal exposure of athymic (nu/nu) BALB/c ice to influenza virus leads to a persistent infec-on of the respiratory tract from which the mice e, usually within 3 to 4 wk with symptoms of neral cachexia. However, if these nude mice were jected 1 day after infection, with approximately)[6] cells from individual virus-specific MHC class II-stricted Th cell clones, they showed greatly re-ced mortality and the titers of infectious virus in eir lungs were reduced, often to undetectable lev-s. By coinfecting mice with pairs of antigenically stinct viruses and subsequently determining the tent of clearance of each type of virus, it could be own first that the clearance mechanism was im-unologically specific but did not display the typi-l crossreaction of class I-restricted cytotoxic T c) cells. In addition, neither primary nor memory : responses could be detected in these mice. Sec-nd, Th cell clones promoted clearance solely of ose viruses that contained the specific Th cell terminant, i.e., Th cell-nonreactive bystander vi-ses were not cleared. These findings were com-tible with virus clearance being effected either rectly after recognition of infected class II-positive lls by the transferred Th cells or indirectly via romotion of a glycoprotein-specific antibody re-onse. The latter seems to be the case because ansfer of Th cells into infected T and B cell-defi-ent SCID mice did not result in virus clearance, though transfer of an anti-hemagglutinin anti-dy cocktail did. Thus, a virus-specific Tc cell re-onse is not a requirement for recovery from a ulmonary influenza virus infection.

MHC class I-restricted Tc[4] cells are thought to play an portant role in the recovery from virus infections (1. . In the case of influenza virus infection, this notion is ased on several lines of evidence: 1) adoptive transfer of spleen cells enriched for virus-specific effector Tc cells into mice shortly after pulmonary infection with influenza virus was found to result in a reduction in mortality and lung virus titer compared to similarly infected normal mice, and the beneficial effect of the transferred cell preparations correlated with their titer in Tc cell activity (3, 4). The same was true when Tc cell clones were used for adoptive transfer instead of heterogeneous, and thus less precisely characterizable, spleen cell preparations (5–8). 2) With a notable exception (9), some studies have indicated that virus-neutralizing antibodies, although effective in preventing an infection when present at the time of exposure to virus (10–12), were not capable of clearing the disease on their own when given (or actively produced) after establishment of the infection (4, 13). 3) B cell suppressed (14) or Th cell-suppressed mice (15, 16) recovered from infection despite a strongly reduced or undetectable anti-viral antibody response. Taken together, most previous studies appeared consistent with the idea that Tc cells were indeed required for clearance of a pulmonary influenza virus infection in mice. Most recently, however, Eichelberger et al. (17) reported that β2-microglobulin-negative mice, which are devoid of class I-restricted T cells, recovered normally from a pulmonary influenza virus infection. Thus, although the Tc cell response may make an important contribution to the recovery of normal virus-infected mice, it can apparently be substituted by other defense mechanisms (2).

In the course of a previous study (18), in which we investigated the function of class II-restricted Th cells in promoting antiinfluenza virus B cell responses in vivo, we observed that adoptive transfer of individual Th cell clones into influenza virus-infected athymic (nu/nu) BALB/c mice resulted in significant reduction, often to undetectable levels, of virus in the lung. We have further investigated this phenomenon and show that class I-restricted Tc cells are not involved in the observed virus clearance and that anti-HA antibodies, on their own, can mediate clearance of influenza virus from the lung.

MATERIALS AND METHODS

Mice. Euthymic female BALB/cbyJ were obtained from The Jackson Laboratories, Bar Harbor, ME and athymic (nu/nu) BALB/c mice were obtained from Harlan Sprague-Dawley, Indianapolis, IN. SCID mice (19) were obtained from the breeding colony of the Wistar Institute.

Viruses and antibodies. The influenza viruses were grown in the allantoic cavity of 10-day-old embryonated hen's eggs. PR8 (A/PR/8/34 (H1N1)) and the reassortant virus X31 (PR8 × A/Aichi/68 (H3N2)) (20) belong to influenza type A; they differ from each other solely by HA and NA, which are of H1 and N1 subtypes in PR8, but of H3 and N2 subtypes in X31. These viruses do not cross-react at the level of virus-neutralizing and neuraminidase-inhibiting antibodies. B/Lee/40 belongs to influenza type B and is immunologically noncross-reactive with type A viruses. Most of the mAb used here have been described previously. Ascitic fluids from hybridon

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[4] Abbreviations used in this paper: Tc cell, cytotoxic T cell; HA, hemgutinin; M, matrix-protein; NA, neuraminidase; MID_{50}, mouse infec-s dose 50%; Iac-BSA, Iscove's modified Dulbecco's medium supple-ented with gentamicin and 0.1% BSA; MDCK, Madin Darby canine ney.

grown in the peritoneal cavity of retired BALB/c breeder mice were used in all cases and were used, within each group, as pools to avoid selection of viral escape mutants (21). The H1-subtype HA (PR8)-specific antibody cocktail contained mAb Y8-2C6 (IgM), Y8-3B3 (IgG3), H28-A2 (IgM), H35-C7 (IgG2a), H37-87 (IgG1), and H37-66 (IgA). The H3-subtype HA(X31)-specific antibody cocktail contained H14-A20 (IgG2b) and H14-A21 (IgG1). The HA (B/Lee)-specific antibody cocktail contained 1.2C7, 1.5A4, 1.1B8, and 1.2F4, all of IgG2a isotype.

T cell clones. The T cell clones were generated from the spleen or lymph nodes of PR8-primed BALB/c mice and were maintained in vitro as described (22, 23). The cells recognize antigen in association with MHC class-II molecules, are $CD4^+/CD8^-$, and secrete IL-2 upon stimulation. The Ag-specificity and class-II isotype restriction of these clones is as follows: T2.5-26 (matrix-protein (M), I-A^d) (22), V1.2 (site 1 of HA, I-E^d) (20), 5.1-7 (neuraminidase (NA), I-E^d) (19), 7.1-5 (site 4 of HA, I-E^d) (23), and 7.1-6 (nucleoprotein, I-E^d). T cells used in adoptive transfer studies were harvested from maintenance cultures 10 to 20 days after Ag stimulation. BEfore transfer they were centrifuged onto a Ficoll-Hypaque cushion (10 min at 900 × *g* at room temperature) to remove dead cells and debris. Cell viability was determined by trypan blue exclusion.

Virus infection. The 5- to 7-wk-old mice were infected with a dose of aerosolized virus corresponding to 100 MID_{50} as described (22). After 18 h, the nude mice were reconstituted by i.v. injection with 1 to 2 × 10^6 Th cells.

Determination of lung virus titers. Lungs were aseptically removed from animals at various intervals after infection, rinsed in sterile PBS, and stored at -20°C until assay. At the time of assay, individual lungs were disrupted with Dounce homogenizers in 2 ml ice-cold PBS containing 0.1% BSA. The extracts were centrifuged for 10 min at 750 × *g* to pellet cell debris and the supernatants diluted serially 10-fold in Isc-BSA. A total of 100 μl of a freshly trypsinized suspension of MDCK cells (5 × 10^5/ml Isc-BSA) were added to wells of flat-bottomed 96-well microtiter plates followed by addition of 50 μl of lung-extract dilutions to four to six replicate wells. The cultures were incubated overnight at 37°C in humidified air/7% CO_2. A total of 50 μl of trypsin (2.5% trypsin) (Whittaker Bioproducts, Inc., Walkersville, MD, freshly diluted 1/750 in Isc-BSA) was then added to each well and the cultures were further incubated as above. After 3 days of incubation, the culture supernatants were tested for the presence of viral HA activity by mixing 25 μl of supernatant with 25 μl of a 1% suspension of chicken RBC. Lung virus titers are expressed as dilution of lung extract at which 50% of the MDCK cultures revealed virus growth ($TCID_{50}$). In some experiments, undiluted lung extracts were inoculated into the allantoic cavity of 10-day-old embryonated hen's eggs. The eggs were incubated at 35°C for 3 days and allantoic fluid was tested for presence of HA activity as above.

To determine the titer of an individual virus strain in lungs of mice coinfected with two virus strains, a 1/10 dilution of the lung extract was pre-incubated for 1 h at 4°C with a 1/100 dilution of a mixture of mAb (ascites fluids) specific for the HA of the virus to be neutralized. The extract was then serially 1/10 diluted in Isc-BSA and tested for residual infectious virus as above.

Isolation of primary Tc effectors. Trachea and attached lungs were lifted out of the thoracic cavity, rinsed briefly in PBS, and transferred into a petri dish. The lungs were lavaged with sterile PBS using a tuberculin syringe with 22-gauge needle inserted into the trachea. The cells released were collected and stored on ice. The lung tissue was minced with scissors and the fragments incubated for 30 min at 37°C in 2 ml Iscove's medium containing 2 mg/ml collagenase (isolated from *Clostridium hystolyticum*, Boehringer Mannheim, Indianapolis, IN). Cells released via both methods were pooled, washed twice, and used as effectors in a cytotoxicity assay. Cell recovery was in general 2 to 10 × 10^6 lymphocytes/lung. To test for primary Tc effectors in the spleens of virus-infected mice, the spleen was removed, a single cell suspension prepared, and the cells washed twice and used in the cytotoxicity assay.

Secondary Tc cultures. Spleens were removed from mice 30 to 60 days after virus infection. Sixty million responder splenocytes were cultured with 3 × 10^6 splenic stimulator cells (2200 rad) and infected with PR8 in 45 ml Iscove's medium supplemented with gentamicin and 5% FCS. After 6 days, the cells were washed, and viable cells counted and tested in the cytotoxicity assay.

Cytotoxicity assay. Cytotoxicity was assayed by ^{51}Cr release assay essentially as described by Bennink et al. (24). In brief, virus-infected or uninfected P815 ($H\text{-}2^d$), BW5147 ($H\text{-}2^k$), and YAC cells were labeled with ^{51}Cr, washed, resuspended at 10^5 cells/ml in Iscove's + 5% FCS, and distributed into round-bottomed microtiter plates at 100 μl/well. A total of 100 μl of effector cells was added to give E:T ratios of 30:1, 10:1, 3:1, and 1:1. After 5 h of incubation at 37°C in air/7% CO_2, 100 μl of supernatant were removed and the amount of ^{51}Cr released determined in a gamma counter (LKB Instruments, Inc., Rockville, MD). Results are presented as percentage specific release, which is defined as (experimental release-spontaneous release)/(total release − spontaneous release) × 100. Total release is the cpm obtained by lysis of target cells with 1% SDS. Spontaneous release (from targets in absence of effectors) was always less than 20% of the total release in these assays.

HI test. Twenty-five μl of serum samples were serially 1/2 diluted in round-bottomed polystyrene microtiter plates and 25 μl of virus corresponding to four agglutinating doses in this assay were added to each well. After 1 h incubation at room temperature, 50 μl of 1% chicken RBC were added to each well, the plates briefly vortexed, and the pattern of agglutination of the RBC recorded after 40 min. The HI test titer was expressed as reciprocal of the serum dilution at which three of the four agglutinating doses of virus were inhibited from agglutinating the RBC. The diluent used in these assays was PBS containing 0.04% NaN_3.

RESULTS

PR8-infected nude mice show significant reduction in mortality and lung virus titer after adoptive transfer of virus-specific Th cell clones. Upon intranasal infection with a sublethal dose of influenza virus, immunocompetent mice develop a temporary viral pneumonia that is resolved within 7 to 10 days with apparently complete clearance of infectious virus from the lungs (Fig. 1*A*). In similarly infected nude mice, the pulmonary infection persists until the mice die of cachexia (Fig. 1*B*); thus, nude mice are incapable of generating effective defense mechanisms that can resolve the pulmonary infection. These observations confirm previous reports and clearly show that T cells are required for recovery from pulmonary influenza virus infection (25, 26).

To investigate the role played by Th cells in induction and expression of these antiviral defense mechanisms, individual PR8-specific Th cell clones were transferred i.v. into nude mice 1 day after infection and titers of infectious virus in lung extracts were determined at various times thereafter in the MDCK infectivity assay. As shown in Figure 1, *C–F*, transfer of individual Th cell clones specific for any one of the four major viral structural proteins resulted in significant reduction of pulmonary virus titers compared to nonreconstituted nude mice (Fig. 1*B*). However, in some of the reconstituted mice, infectious virus appeared to persist in the lung at low levels, becoming detectable only after inoculation of undiluted lung extract into the allantoic cavity of embryonated eggs. In this manner, residual infectious virus was revealed in five of eight lung extracts from reconstituted mice that had scored negative in the MDCK assay, whereas no virus was isolated from four lung extracts of reconvalescent euthymic mice. The M-specific Th cell clone was most effective by promoting apparently complete virus clearance in roughly half of the recipients.

The beneficial effects of the transferred Th cell clones was evidenced also by reduction of mortality. To accelerate death in nonreconstituted nu/nu mice, the challenge dose was increased for these experiments. As shown in Figure 2, infection with 1000 to 5000 MID_{50} of PR8 resulted in 100% mortality by day 20 in nonreconstituted nude mice. In contrast, transfer of the HA- or M-specific Th cells prevented mortality in all mice, and most animals displayed no signs of illness as long as 3 mo postinfection.

Specificity of virus clearance. The Th clones included in this study secrete IL-2, IL-3, and γ-IFN, but not IL-4 (26a) upon stimulation in vitro and, therefore, belong to the type 1 subset (Th1) (27). Inasmuch as γ-IFN has inhibitory effects on virus replication (28) and also is a

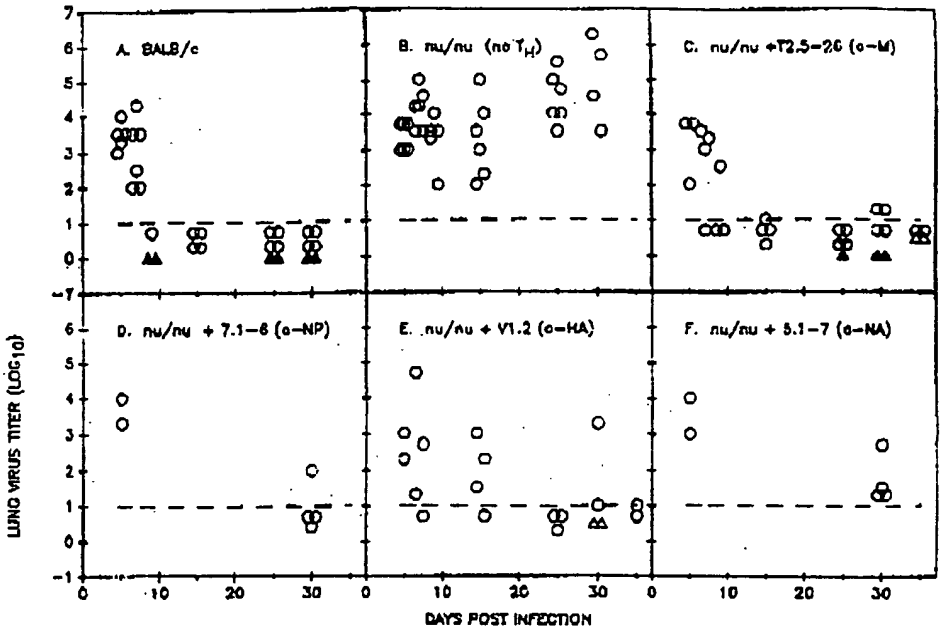

Figure 1. PR8-infected nude mice show partial to complete clearance of virus after transfer of Th cell clones. The data are a compilation of several independent experiments in which normal or athymic mice had been exposed to an aerosol of 100 MID_{50} of PR8 per mouse and, 1 day later, were injected i.v. with 1 to 2 million cells from individual Th cell clones. Lung virus titers were determined at various timepoints after infection in the MDCK infectivity assay (*circles*) and in embryonated eggs (*triangles*). The *stippled horizontal line* indicates the threshold of detectability in the MDCK assay. Virus titers are expressed as $TCID_{50}$ per 25 μl of lung extract. Selected lung extracts that contained no detectable virus in the MDCK assay were retested undiluted in embryonated eggs and are displayed as *open triangles* when found to be positive and *closed triangles* when found to be negative.

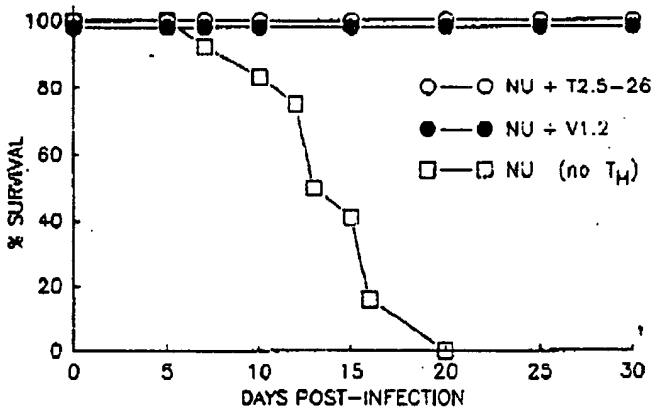

Figure 2. Th cell transfer reduces mortality of nude mice after exposure to a lethal dose of influenza virus. Nude mice were exposed to an aerosol dose of 1000 to 5000 MID_{50} of PR8 per mouse and were injected i.v. 18 h later with PBS (□) or 1–2 × 10^6 cells from an HA-specific (●) or M-specific (○) Th cell clone. Each group contained 8 to 12 mice at the initiation of the experiment.

TABLE I

Virus clearance in reconstituted nude mice is type specific[a]

Infecting Virus Strains	Transferred Th Cells	Virus Titer in Lung (log 10)	
		A/PR8	B/Lee
[1] A/PR8 + B/Lee		4.9, 4.9, 4.9	3.9, 2.9, 2.9
A/PR8	T2.5-26	<[b], 2.9	
B/Lee	T2.5-26		3.9, 3.9
A/PR8 + B/Lee	T2.5-26	<, <	3.4, 3.65
A/PR8 + B/Lee	V2.1	<, <	3.9, 5.4

[a] Nude mice were exposed to an aerosol of A/PR8 or B/Lee or a mixture of A/PR8 + B/Lee and, 1 day later, injected i.v. with cells from the indicated Th cell clones. Lung extracts, prepared 20 days postinfection, were pretreated with mAb cocktails that neutralized A/PR8 or B/Lee, respectively, to determine the titers of each virus strain separately in each extract. The MDCK infectivity assay was used and virus titers are expressed as $TCID_{50}$ per total lung extract. Virus titers from individual experimental mice are shown.

[b] < indicates no detectable virus at 1/10 dilution of extract; corresponds to a titer per total extract of <2.1.

strong activator of nonspecific effectors such as NK cells and macrophages (29), we were interested in determining whether these nonspecific mechanisms played a role in virus clearance. The approach we used was to assess virus clearance in nude mice that had been coinfected with PR8 and B/Lee and were reconstituted with a PR8-specific Th cell clone. Titers of each individual virus were then determined in the MDCK assay after preincubation of the lung extracts with a cocktail of virus-neutralizing mAb specific for either PR8 or B/Lee. Inasmuch as B/Lee virus does not cross-react with the type A virus PR8 at neither B nor T cell level, clearance of B/Lee in these coinfected mice would implicate nonspecific mechanisms. As shown in Table I, PR8 and B/Lee grew to clearly detectable titers in the lungs of coinfected nude control mice and both viruses were cleared from the lungs of coinfected immunocompetent BALB/c mice (data not shown). After transfer of the Th cell clones V1.2 or T2.5–26 into coinfected nude mice, PR8 became cleared to a nondetectable level, while no reduction in B/Lee titer was seen.

Virus reduction in reconstituted nude mice occurs in absence of detectable Tc cell response. Although nude mice at a young age are essentially without T cell function, they have been shown to regain some T cell functions upon treatment with IL-2 (30–32). Inasmuch as the transferred Th cells produce IL-2 after Ag stimulation in vitro and could be expected to do so also upon activation in vivo, it was possible that endogenous Tc cells were generated in the Th cell-reconstituted nude mice and that they, in turn, were responsible for the observed virus clearance. To test this possibility we made use of the fact that the vast majority of Tc cells induced in mice by infection with PR8 is directed against determinants derived from internal and nonstructural viral proteins (33). Accordingly, nude mice were coinfected with PR8 and X31, the latter being a reassortant virus that differs from PR8 solely by its HA and NA that are of the H3 and N2 subtypes, respectively. Thus, promotion of a Tc cell response by the transferred Th cell clone would be expected to lead to simultaneous clearance of both viruses. However, as shown in Table II, transfer of the HA (PR8)-

TABLE II

Virus clearance in Th cell-reconstituted nude mice is subtype specific[a]

Infecting Virus Strains	Th Transferred	Lung Virus Titers (log 10)		Serum HI Titers Vs	
		PR8	X31	PR8	X31
PR8 + X31		4.6, 4.1	5.3, 5.1	1/80	—
PR8	7.1-5 (a-PR8)	<, <, <, <[b]		*1/640*[c]	—
X31	7.1-5 (a-PR8)		5:1, 4.9, 4.1, 4.3	—[d]	—
PR8 + X31	7.1-5 (a-PR8)	<, <, <, <	3.6, 4.3, 2.9, 4.9	*1/160*	—
PR8 + X31	T2.5-26 (cross-reactive)	<, <	<, 2.3	*1/480*	*1/640*

[a] The coinfection experiments were performed as indicated in the legend of Table I except for the use of the type A reassortant virus X31 instead of B/Lee. Virus titers were determined in lung extracts prepared from mice at 20 or 30 days after infection. Virus titers from individual experimental mice are shown. HI titers show the maximum mean titer observed in two sera obtained at 8 or 20 days after infection.

[b] < indicates no detectable virus at 1/10 dilution of extract; corresponds to a titer per total extract of <2.1.

[c] Sera with significant increases (≥fourfold) in HI titers compared to nonreconstituted nude mice are shown in italics.

[d] Titers do not exceed inhibition observed with sera from naive mice. The threshold of detection was 1/40 for PR8 and 1/120 for X31. Sera were tested without prior inactivation of preexisting nonspecific inhibitors.

TABLE III

Th cell-reconstituted nude mice do not contain virus-specific Tc cell precursors in spleen after recovery from virus infection[a]

Responder Cultures	E/T ratio	Percent Specific ^{51}Cr Release		
		P815-PR8	P815-B/Lee	P815
BALB/c	30:1	78	12	3
BALB/c	10:1	64	6	8
BALB/c	3:1	44	2	2
BALB/c	1:1	30	0	0
nu/nu	30:1	−2	ND	1
nu/nu + Th	30:1	0	ND	0
nu/nu + Th + CAS[b]	30:1	7	ND	4

[a] Splenocytes obtained from experimental mice 30 days after infection with PR8 were restimulated in vitro with PR8 and tested after 6 days of culture at the indicated E:T ratios for the presence of virus-specific cytotoxic activity by ^{51}Cr release assay. Spontaneous release was 19% for P815-PR8, 10% for P815-B/Lee and 13% for uninfected P815 cells. The experimental mice were euthymic BALB/c or nonreconstituted (nu/nu) and Th cell-reconstituted (nu/nu + Th) athymic BALB/c mice.

[b] The culture was supplemented with supernatant from Con A-stimulated rat spleen cells (CAS) at a final concentration of 2% to provide Th cell-derived factors during restimulation in vitro.

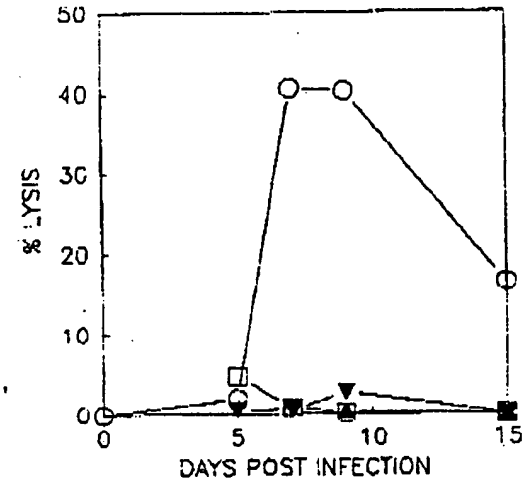

Figure 3. No virus-specific Tc cell activity can be detected in the lungs of infected and Th cell-reconstituted nude mice. Mice were infected by exposure to a PR8 aerosol and cells isolated from the lungs of nude mice not reconstituted (□), nude mice reconstituted with 1 to 2 × 10^6 cells of the M-specific (▼) or HA-specific (▲) Th cell clones, and normal mice (○) were assayed for PR8-specific cytotoxic activity by Cr-release assay. The data show the percentage specific lysis observed at a 30:1 E:T ratio vs PR8-infected P815 cells. Lysis of uninfected cells was less than 4% in all cases.

specific Th cell clone 7.1–5 resulted in clearance of PR8 only.

Both viruses were cleared, however, after transfer of the M-specific Th cell clone T2.5–26 that recognizes both PR8 and X31, as they have the M protein in common. Although this finding would in principle be consistent with an involvement of Tc cells in this specific transfer experiment, a more likely explanation is that the induction of an antibody response to the viral glycoproteins is involved. The production of an antibody response to the viral glycoproteins in this transfer system requires that the protein recognized by the Th cell be located within the same virus structure for a successful T-B collaboration to occur (19). Accordingly, the PR8-specific Th cell clone 7.1–5 promoted an anti-HA response to PR8 alone while the cross-reactive Th cell clone T2.5–26 promoted anti-HA responses to both viruses (Table II).

Two additional types of experiments were performed in search of a virus-specific Tc cell response in this transfer system. First, experimental mice were tested 30 days after infection for the presence of virus-specific memory Tc cells by restimulation of splenocytes in vitro. As shown in Table III, a high level of virus-specific Tc cell activity was obtained with splenocytes from normal BALB/c mice, yet none could be detected in spleen cell cultures from nude mice, even when additional T cell-derived factors (Table III) or irradiated splenocytes from PR8-immune mice (data not shown) were added to the stimulation cultures. Second, cell preparations isolated from the lungs of experimental mice at various time points after infection were tested directly for Tc cell activity. Again, only cells recovered from infected euthymic BALB/c mice displayed significant Tc cell activity, which reached maximum levels in the lung at 7–10 days after infection (Fig. 3).

Virus can be cleared by passively transferred anti-HA antibodies. Taken together, the coinfection experiments had shown that adoptively transferred Th cells promoted clearance only of the virus(es) for which they displayed specificity. This was consistent with clearance resulting either from a direct interaction between Th cells and infected lung cells, or the promotion of an antiviral antibody response that, as mentioned above, had been shown to be dependent on the presence of Th and B cell determinants within the same virion. To distinguish between these possibilities we transferred Th cells, anti-HA antibodies, or a combination of Th cells plus antibodies into PR8-infected B cell- and T cell-deficient SCID mice. As shown in Table IV, transfer of antibody but not of Th cells resulted in virus clearance. Titration of anti-HA antibodies into PR8-infected SCID and nude mice further showed (Table V) that the serum antibody titers at which clearance was achieved are in the same range as those observed in the Th cell-reconstituted nude mice (compare Tables II and V).

TABLE IV

Effects of anti-HA antibodies and Th cells on virus clearance in SCID mice[a]

Treatment	Serum HI Titer	Virus Titer in Lung (log 10)
	<1/50	7.4, 7.3, 7.4
Th (day 1)	<1/50	6.1, 6.4, 6.0, 6.1
Th (day 1) + antibody	1/2800	<[b], <, <, <
Antibody (days 2 and 6)	1/2800	<, <, <, <

[a] SCID mice were infected by exposure to a PR8 aerosol and separated into four treatment groups. One day postinfection, two groups were injected i.v. with 2×10^6 cells from Th cell clone T2.5-26, and 2 and 6 days postinfection, two treatment groups were injected i.p. with 0.25 ml of undiluted PR8 HA-specific antibody cocktail. All mice were killed at day 20 postinfection and virus titers and HI titers were measured in lung extracts and sera, respectively. Lung virus titers of individual mice and mean HI titers are shown.

[b] < indicates no detectable virus at 1/10 dilution of extract; corresponds to a titer per total extract of <2.1.

TABLE V

Effect of anti-HA antibody dose on virus clearance in SCID and nude mice

Mice[a]	Antibody Dose	Virus in Lung (Day 20)		Serum HI Titer[d]
		Number[b] (infected/total)	Virus Titer[c] (log 10)	
SCID		3/3	6.6	<1/20
SCID	1/25	4/4	5.7	1/200
SCID	1/5	3/4	<[e]	1/800
SCID	Undiluted	0/4	<	1/3150
nu/nu		3/3	5.2	<1/40
nu/nu	1/25	4/4	5.3	1/100
nu/nu	1/5	3/4	<	1/500
nu/nu	Undiluted	0/4	<	1/2500

[a] SCID and nu/nu BALB/c mice were infected by exposure to a PR8 aerosol. On day 2 and day 6 postinfection, groups of mice were injected i.p. with 0.25 ml of virus-neutralizing antibody cocktail at the indicated dilutions, or in the control group with PBS. All mice were killed at day 20.

[b] All lung extracts that were negative in the MDCK assay were injected undiluted into the allantoic cavity of two embryonated eggs that were tested 3 days later for virus growth. A lung extract was considered free of infectious virus if virus failed to grow in both eggs.

[c] Lung extracts were tested for virus titer in MDCK assay, starting at a 1/10 dilution. Geometric mean titers are shown.

[d] HI titers vs PR8 were determined in sera and are shown as geometric mean titers of three to four mice per group.

[e] < indicates no detectable virus at 1/10 dilution of extract; corresponds to a titer per total extract of <2.1.

To exclude the possibility that passively transferred anti-HA antibodies merely masked a residual infection but did not truly eliminate it, we maintained PR8-infected and antibody-treated SCID mice for 75 days. By this time, anti-HA antibody titers in serum had decayed virtually to background level, yet the lung extracts remained negative for virus (data not shown). Three similarly infected SCID mice that had not received antibody treatment died within 3 wk after infection.

DISCUSSION

It is well established that virus-neutralizing anti-HA antibodies, if present in sufficient concentration at the site of virus-exposure before challenge, can protect against infection with influenza virus (10–12), and it is generally accepted that antibodies to HA (34), NA (35), and M2 (36) help in limiting virus replication and spread once pulmonary infection has been established. Less clear, however, is whether anti-HA antibodies (which are the only antibodies with virus-neutralizing activity) are capable of clearing an ongoing virus infection in the absence of a virus-specific T cell response. Kris et al. (13), for example, reported that treatment of virus-infected nude mice with virus-neutralizing antiviral antisera lead to only a temporary decrease but not to a permanent cure of the disease. A similar conclusion was reached by Wells et al. (4), who observed that adoptive transfer of a spleen cell preparation with low Tc cell-, but high antibody-secreting activity did not clear the infection in nude mice, in contrast to spleen cells with high Tc cell- and low antibody-secreting activity. However, by studying the course of influenza infection in anti-μ-, anti-γ-, or anti-α-treated mice, Iwasaki and Nozima (37) concluded that antiviral antibodies of IgA isotype were actually required for recovery from disease (a conclusion that was not supported, however, by a similar study performed by Kris et al. (14)). Furthermore, Virelizier (9) showed that treatment of infected thymectomized and bone marrow-reconstituted mice with rabbit anti-HA antiserum reduced virus titers in the lung by day 6 to undetectable level (in which case however, masking of residual infection was not excluded). Our study clarifies this issue by demonstrating a permanent cure of infected SCID mice after treatment with anti-HA antibody.

The reason for the contrasting results obtained in the study of Kris et al. (13) and the present one is not known. Although both studies used similar protocols, there are several differences that could play a role in determining the extent of virus clearance. These include 1) differences between the viruses used, particularly in terms of fine tissue tropism and virulence; 2) the difference in the timing of antibody transfer that was performed on day 5 in Kris' study vs days 2 and 6 here; and 3) differences in the composition or titer of the transferred antibodies. In this context, it is noteworthy that the anti-HA antibody mixture used here contained a polymeric IgA antibody that has been shown to be transported efficiently from serum into respiratory tract secretions (38).

Inasmuch as transfer of Th cells into infected SCID mice did not cure the disease, we conclude that the Th cells, after transfer into infected nude mice, promoted recovery indirectly by supporting an antiviral B cell response. We have shown previously that Th cells specific for each of the major viral proteins promote an antibody response to both HA and NA in this system (19). It is possible also that the transferred Th cells aided in recovery through the promotion of additional mechanisms. For example, the Th cells may have enhanced antibody-dependent cell-mediated cytotoxic effector functions through IL-2- and γ-IFN-mediated activation of the corresponding effector cells. Th cells may additionally have contributed to the ultimate antibody-dependent recovery by limiting (although not stopping) early stages of the infection by lysis of infected class II$^+$ lung cells or through nonspecific mechanisms such as secretion of γ-IFN and TNF or activation of NK cells and macrophages (37, 39, 40). With regard to Th-mediated cell lysis, it is noteworthy, however, that only two of the Th included in this analysis (V2.1 and V1.2) showed significant ^{51}Cr-release (34 and 36% at E/T ratio of 30/1) against virus-pulsed A20 cells, whereas the others were essentially negative (9% specific lysis at E/T ratio of 30/1 for 5.1–7 and 7.1–5 and 3% for T2.5–26 and 7.1–6). However, as shown by the persistence of virus in the lungs of SCID mice reconstituted with Th alone, one must conclude that these latter mechanisms are not capable of curing the disease on their own. The possibility that the transferred Th were not activated in the infected SCID mice in the absence of B cells appears unlikely.

In a study similar to the one reported here, in which

Th cell clones were transferred into mice 1 to 2 h after intranasal infection with a sublethal dose of influenza virus X31. Taylor et al. (41) found that Th cells had variable effects on the course of infection, with some resulting in an accelerated virus clearance although others exacerbated morbidity. The differences in "disease-curing" activity between Th cell clones observed in this study are less dramatic than those reported by Taylor et al. (41). Thus, of 18 distinct Th cell clones of type 1 that we have tested by transfer into infected nude mice (present results and our unpublished data), only 4 failed to promote virus clearance (less than 100-fold reduction in lung virus titer 10 to 15 days after infection compared to nonreconstituted nude mice) and none exacerbated the disease. It is possible that the increased morbidity seen in some instances by Taylor et al. (41) is the reflection of an excessive Th cell-induced inflammatory response that we may not have seen because we transferred 2 to 10 times fewer Th cells per recipient and used nude instead of normal recipient mice.

In conclusion, the present study shows that although Tc cells are likely to make a significant contribution to the recovery of fully immunocompetent mice from pulmonary influenza virus infection, they are not absolutely required for this purpose and, when defective, can be substituted by antibodies or Th cell-dependent mechanisms.

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